UNIVERSITI PUTRA MALAYSIA

L-PHENYLALANINE PRODUCTION BY STABILISED CALCIUM ALGINATE IMMOBILISED AMINOACYLASE

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L-PHENYLALANINE PRODUCTION BY STABILISED CALCIUM ALGINATE IMMOBILISED AMINOACYLASE

By

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LIST OF ABBREVIATIONS

ACAG	Calcium Alginate Immobilised Aminoacylase
GLU	Glutaraldehyde
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
K_m	Michaelis-Menton Constant
PEI	Polyethyleneimine
PLL	Poly-L-Lysine
Tris	Tris(hydroxymetyl)-aminomethane
V_{max}	Maximal Velocity

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Faculty : Food Science and Biotechnology

Enzymatic resolution of DL-amino acids using aminoacylase immobilised on DEAE-sephadex has been employed in the industry for the production of the optically active, L-amino acid. There are, however, some limitations to this technique. The aims of this study were to establish an efficient immobilisation method for aminoacylase and to report the preparation, properties, and applicability of the resulting immobilised aminoacylase. Aminoacylase was immobilised in calcium alginate beads (ACAG). The ACAG was further stabilised by coating with polyethyleneimine (PEI), a mixture of polyethyleneimine and glutaraldehyde (PEI-GLU), poly-L-lysine (PLL), or by cross-linking with carbodiimide. Each type of the immobilised aminoacylase was then used to perform the optical resolution of N-acetyl-DL-phenylalanine. PLL-coated ACAG

was found to be the best among the immobilised aminoacylase studied. It has a higher activity and stability than that of the other systems. In the batch operational stability study, half of the ACAG activity was lost after the first cycle of reaction. The cross-linked ACAG has a half-life of four reaction cycles while the PEI-coated, PEI-GLU-coated ACAG and PLL-coated ACAG were stable up to ten cycles of reaction. Although the stability of ACAG was enhanced by coating with PEI and PEI-GLU, the specific activity of PEI & PEI-GLU-coated ACAG were greatly reduced when compared to that of ACAG. The activity of ACAG, however, was not decreased significantly after coating with PLL. Electron micrographs of the uncoated ACAG and coated ACAG revealed some differences in their surface structures. The surface of ACAG was smooth while that of PEI and PLL-coated ACAG had a cross-linked network. The cross-linked ACAG, however, had the same surface structure as ACAG. In the continuous operational stability study, the packed-bed bioreactors containing PEI and PLL-coated ACAG were stable for 25 days.

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PENGHASILAN L-FENILALANINA OLEH AMINOASILASE TERSEKAT-GERAK DALAM KALSIUM ALGINAT YANG STABIL

Oleh

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Resolusi menggunakan enzim aminoasilase tersekat-gerak pada DEAE-sephadex telah digunakan dalam industri untuk menghasilkan asid amino yang aktif secara optikal. Walau bagaimanapun, terdapat beberapa kelemahan dalam teknik ini. Tujuan penyelidikan ini adalah untuk mendapatkan sistem enzim tersekat-gerak yang unggul untuk aminoasilase dan melaporkan cara-cara penyediaan, ciri-ciri, dan kegunaan sistem enzim tersekat-gerak yang terhasil. Aminoasilase telah disekat-gerak dalam manik kalsium alginat (ACAG). ACAG itu kemudian distabilkan dengan penyalutan oleh polietilenaimina (PEI), campuran polietilenaimina dan glutaraldehida (PEI-GLU), poli-L-lisina (PLL) atau ikatan silang dengan karbodiimida (ACAG terikat silang). Aminoasilase tersekat-gerak yang terhasil digunakan untuk melakukan tindakbalas resolusi secara optikal ke atas N-asetil-DL-fenilalanina. ACAG yang disaluti dengan

PLL merupakan sistem enzim tersekat-gerak yang terbaik di antara yang diselidiki. Ia lebih aktif dan stabil daripada sistem yang lain. Di dalam kajian kestabilan operasi sesekelompok, separuh daripada aktiviti ACAG telah hilang selepas tindakbalas yang pertama. ACAG terikat silang mempunyai separa hayat selama empat kitaran tindakbalas sementara ACAG yang disaluti PEI, PEI-GLU, dan PLL masing-masing stabil sehingga sepuluh kitaran tindakbalas. Walaupun kestabilan ACAG telah meningkat melalui penyalutan dengan PEI dan PEI-GLU, tetapi aktiviti spesifik mereka telah menurun dengan banyak berbanding dengan aktiviti ACAG. Sebaliknya, aktiviti ACAG tidak banyak berubah selepas disaluti dengan PLL. Mikrograf elektron untuk ACAG yang tidak disaluti dan yang telah disaluti menunjukkan perbezaan dalam struktur permukaan mereka. Permukaan ACAG adalah licin sementara ACAG yang disaluti dengan PEI dan PLL mempunyai struktur rangkaian ikatan silang. Walau bagaimanapun, ACAG terikat silang mempunyai struktur yang sama seperti ACAG. Di dalam kajian kestabilan operasi secara berterusan, bioreaktor "packed-bed" yang mengandungi ACAG yang disaluti dengan PEI dan PLL telah didapati stabil selama 25 hari.

CHAPTER I

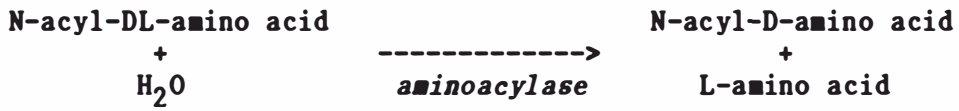
GENERAL INTRODUCTION

The world demand for amino acids is vast; 675 tons of amino acids consumed in 1985. It represents an increase of 34 % in volume from 1981 to 1985 (Kinoshita, 1987). The turnover was estimated at 1.7 billion U.S. dollars worldwide in 1980 (Crueger and Crueger, 1984). All the twenty amino acids that are required for the synthesis of protein have been produced industrially with L-glutamic acid being the most, followed by D,L-methionine and L-lysine. Today, the manufacture of amino acids is largely dominated by the Japanese and there is hardly any such industry in Malaysia except for the production of monosodium glutamate (MSG) by Ajinomoto Co..

Amino acids are used in medicine to strengthen the defence mechanism, to increase blood coagulation, for gluconeogenesis, and for synthesis of new protein in hospitalized patients. In food industry, amino acids are added to foods to enhance the taste, flavour as well as quality of the foods. A new application of amino acids in foods recently is as a low calorie sweetener, aspartame (L-aspartyl-L-phenylalanine methyl ester), which consists of L-phenylalanine and L-aspartic acid.

Amino acids such as lysine and methionine have been added to feedstuff to upgrade it.

Industrial production of amino acids is performed by fermentative and chemical synthetic methods. The latter method is more simple, faster, and cheaper to perform than the former method. Nevertheless, the amino acids produced chemically are the optically inactive racemic mixture of D- and L-isomers. The L-isomer is a physiologically active natural form, the D-isomer is generally inactive form for human nutrition, and is an unnatural form. In order to obtain the L-amino acid, optical resolution is necessary. Optical resolution of racemic amino acids has been carried out by physico-chemical, chemical, biological, and enzymatic methods. The enzymatic resolution using aminoacylase is the preferred method as it has versatile substrate specificity and absolute stereospecificity. The reaction catalysed by aminoacylase is as follows :



Aminoacylase selectively hydrolyses the N-acyl-L-isomer. The liberated L-amino acid can be separated from the unhydrolysed N-acyl-D-amino acid based on the difference in their solubilities. The N-acyl-D-amino acid can be racemised and the process be repeated.

The aforementioned enzymatic resolution has been employed extensively in the industrial production of L-amino acid. The enzyme reaction was initially performed in batch process using soluble enzyme. The drawbacks of this procedure are (i) the enzyme is impossible to recover after use, and (ii) the enzyme might not be sufficiently stable under the operational conditions. To overcome these disadvantages, extensive work has been carried out to produce L-amino acids using immobilised aminoacylase (Chibata *et al.*, 1972, Morikawa *et al.*, 1976, Sato *et al.*, 1971, Tosa *et al.*, 1967, Weetal and Detar, 1974, Yokote *et al.*, 1975). Chibata and his associates, in their evaluation of 43 different immobilisation methods for aminoacylase, chose DEAE-sephadex bonded enzyme for industrial application because it allowed them to regenerate the packed-bed enzyme reactor's activity every so often (without unloading the reactor), by flushing the system with high salt concentration and low pH to remove the denatured enzyme, subsequently adding fresh enzyme directly to the reactor to allow immobilisation to take place in situ. The original DEAE-sephadex was left in the reactor for at least 10 years. The limitations to this technique are (i) the enzyme must be active within the narrow range of environmental conditions (particularly pH) required to keep the enzyme bonded to the polymer matrix, (ii) some release of aminoacylase may occur if high concentration of substrate is used, (iii) the aminoacylase used may contain other enzymes such as cellulase and dextranase which can hydrolyse DEAE-

sephadex, and (iv) DEAE-sephadex is an expensive ion-exchange resin. Further efforts should thus be made to prepare immobilised aminoacylase which has the aforementioned limitations removed.

The objective of this study in general is to obtain a stable immobilised aminoacylase system for the production of L-phenylalanine via the optical resolution of N-acetyl-DL-phenylalanine. The method of encapsulation of aminoacylase in calcium alginate beads was chosen due to the fact that (i) this immobilisation process is simple and mild, thus the enzyme may be maintained in its native form after immobilisation, and (ii) the matrix is cheap and non-toxic. However, it is known that the abovementioned method is not suitable for immobilisation of enzymes as the pores on the surface of calcium alginate beads are large enough to allow enzymes to diffuse out of the beads. Thus, the specific aim of this study is to prevent aminoacylase from leaking out of the calcium alginate beads. To achieve this, various methods for the stabilisation of the calcium alginate immobilised aminoacylase have to be designed and evaluated.

The first method would involve coating the calcium alginate immobilised aminoacylase with (i) polyethyleneimine, (ii) a mixture of polyethyleneimine and glutaraldehyde, and (iii) poly-L-lysine. It is hoped that by coating the calcium alginate beads with another layer of polymer, a coverage of the